

Amendment and Response

Applicants: Sprecher et al.

Serial No.: 10/800,057

Filed: March 12, 2004

For: NOVEL HUMAN KUNITZ-TYPE INHIBITORS AND METHODS RELATING THERETO

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Reconsideration and withdrawal of the rejections are respectfully requested. Claims 2, 11 and 13 having been canceled and claims 1, 10, 12 and 21 having been amended, the pending claims in the instant application are claims 1, 3-10, 12 and 14-22. No new matter has been added.

Claims 1, 10, 12 and 21 have been amended to include the stringent hybridization conditions as set forth in Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY, 1989, which is incorporated by reference in the specification at page 8, lines 22-24. A copy of the cover page, copyright page and relevant pages which include the hybridization conditions are provided herewith.

Applicants reserve the right to prosecute claims to canceled subject matter in one or more continuing applications.

Obviousness-Type Double Patenting Rejections

The Examiner rejected claims 5-11 and 16-22 under the judicially created doctrine of obviousness-type double patenting as allegedly being unparentable over claims 1-17 of U.S. Patent No. 5,455,338.

Submitted herewith is a Terminal Disclaimer which, Applicants submit, is in compliance with 37 CFR 1.321(c) and thereby obviates the Examiner's double patenting rejection of amended claims 5-10 and 16-22. Accordingly, withdrawal of the obviousness-type double patenting rejection is respectfully requested.

The Examiner rejected claims 1-4 and 12-15 under the judicially created doctrine of obviousness-type double patenting as allegedly being unpatentable over claims 1-6 of U.S. Patent No. 5,914,315.

Submitted herewith is a Terminal Disclaimer which, Applicants submit, is in compliance with 37 CFR 1.321(c) and thereby obviates the Examiner's double patenting rejection of amended claims 1, 3, 4, 12, 14 and 15. Accordingly, withdrawal of the obviousness-type double patenting rejection is respectfully requested.

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The Examiner provisionally rejected claims 1-4 and 12-15 under the judicially created doctrine of obviousness-type double patenting as allegedly being unpatentable over claims 1-6 of copending Application No. 10/680,684 (US 2004/0253686).

Upon an indication of otherwise allowable subject matter and in the event this rejection is maintained, Applicants will provide an appropriate response.

Rejection Under 35 U.S.C. §112, First Paragraph

The Examiner rejected claims 2, 11, and 13 under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. This rejection is respectfully traversed.

In order to advance prosecution of the instant application, claims 2, 11, and 13 have been canceled, thereby rendering the Examiner's rejection moot. Withdrawal of the rejection under 35 U.S.C. §112, first paragraph, is respectfully requested.

Rejections Under 35 U.S.C. §112, Second Paragraph

The Examiner rejected claims 1-22 under 35 U.S.C. §112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. This rejection is respectfully traversed.

The Examiner rejected claims 1, 10, 12 and 21 for reciting the allegedly indefinite phrase of "highly stringent hybridization conditions." Applicants have amended claims 1, 10, 12 and 21 to include the specified stringent hybridization conditions as set forth in the incorporated and provided Sambrook et al. reference. Applicants submit that the inclusion of these conditions renders claims 1, 10, 12 and 21 definite. Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. §112, second paragraph, are respectfully requested.

The Examiner rejected claims 2, 11 and 13 for reciting the allegedly indefinite phrase of "conservative amino acid substitution." In order to expedite

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prosecution of the above-identified application, Applicants have canceled claims 2, 11 and 13, thereby rendering the Examiner's rejection moot. Accordingly, withdrawal of the rejection under 35 U.S.C. § 112, second paragraph, is respectfully requested.

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Summary

It is respectfully submitted that the pending claims 1, 3-10, 12 and 14-22 are in condition for allowance and notification to that effect is respectfully requested. If for any reason the Examiner feels that a telephone conference would expedite prosecution of the application, the Examiner is invited to telephone the undersigned at (206) 442-6540.

Respectfully Submitted,



Brian J. Walsh
Registration No. 45,543

Enclosures:

Terminal Disclaimer for U.S. Patent No. 5,455,338

Terminal Disclaimer for U.S. Patent No. 5,914,315

Amendment Fee Transmittal (in duplicate)

1 Reference

Customer No. 10117
ZymoGenetics, Inc.
1201 Eastlake Avenue East
Seattle, WA 98102

2

COPY

Molecular Cloning

A LABORATORY MANUAL
SECOND EDITION

J. Sambrook

UNIVERSITY OF TEXAS SOUTHWESTERN MEDICAL CENTER

E.F. Fritsch

GENE FICS INSTITUTE

T. Maniatis

HARVARD UNIVERSITY



Cold Spring Harbor Laboratory Press
1989

**Molecular
Cloning**

A LABORATORY MANUAL
SECOND EDITION

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Printed in the United States of America

9 8 7 6 5 4 3 2 1

Book and cover design by Emily Harste

Cover: The electron micrograph of bacteriophage λ particles stained with uranyl acetate was digitized and assigned false color by computer. (Thomas R. Broker, Louise T. Chow, and James I. Garrels)

Cataloging in Publications data

Sambrook, Joseph

Molecular cloning : a laboratory manual / E.F.

Fritsch, T. Maniatis—2nd ed.

p. cm.

Bibliography: p.

Includes index.

ISBN 0-87969-309-6

1. Molecular cloning—Laboratory manuals. 2. Eukaryotic cells—Laboratory manuals. I. Fritsch, Edward P. II. Maniatis, Thomas III. Title.

QH442.2.M26 1987

574.87'3224—dc19

87-35464

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HYBRIDIZATION OF RADIOLABELED PROBES TO IMMORILIZED NUCLEIC ACIDS

There are many methods available to hybridize radioactive probes in solution to nucleic acids immobilized on solid supports such as nitrocellulose filters or nylon membranes. These methods differ in the following respects:

- Solvent and temperature used (e.g., 68°C in aqueous solution or 42°C in 50% formamide)
- Volume of solvent and length of hybridization (large volumes for periods as long as 3 days or minimal volumes for periods as short as 4 hours)
- Degree and method of agitation (continuous shaking or stationary)
- Use of agents such as Denhardt's reagent or BLOTTO to block the non-specific attachment of the probe to the surface of the solid matrix
- Concentration of the labeled probe and its specific activity
- Use of compounds, such as dextran sulfate (Wahl et al. 1979) or polyethylene glycol (Renz and Kurz 1984; Amasino 1986), that increase the rate of reassociation of nucleic acids
- Stringency of washing following the hybridization

Although the choice depends to a large extent on personal preference, we offer the following guidelines for choosing among the various methods available.

1. Hybridization reactions in 50% formamide at 42°C are less harsh on nitrocellulose filters than is hybridization at 68°C in aqueous solution. However, it has been found that the kinetics of hybridization in 80% formamide are approximately four times slower than in aqueous solution (Casey and Davidson 1977). Assuming a linear relationship between the rate of hybridization and the formamide concentration, the rate in 50% formamide should be two to three times slower than the rate in aqueous solution. Both types of solvents give excellent results and neither has a clear-cut advantage over the other.
2. The smaller the volume of hybridization solution, the better. In small volumes of solution, the kinetics of nucleic acid reassociation are faster and the amount of probe needed can be reduced so that the DNA on the filter acts as the driver for the reaction. However, it is essential that sufficient liquid be present for the filters to remain covered at all times by a film of the hybridization solution.
3. Continual movement of the probe solution across the filter is unnecessary, even for a reaction driven by the DNA immobilized on the filter. However, if a large number of filters are hybridized simultaneously, agitation is advisable to prevent the filters from adhering to one another.
4. The kinetics of the hybridization reaction are difficult to predict from theoretical considerations, partly because the exact concentration of the

immobilized nucleic acid and its availability for hybridization are unknown. When using probes that have the capacity to self-anneal (e.g., nick-translated double-stranded DNA), the following rule of thumb is useful: Allow the hybridization to proceed for a time sufficient to enable the probe in solution to achieve $1-3 \times C_0 t_{1/2}$. In 10 ml of hybridization solution, 1 μ g of a probe of 5-kb complexity will reach $C_0 t_{1/2}$ in 2 hours. To determine the time of half-renaturation for any other probe, simply enter the appropriate values into the following equation:

$$1/x \times y/5 \times z/10 \times 2 = \text{number of hours to achieve } C_0 t_{1/2}$$

where x = the weight of the probe added (in micrograms), y = its complexity (for most probes, complexity is proportional to the length of the probe in kilobases), and z = the volume of the reaction (in milliliters).

After hybridization to $3 \times C_0 t_{1/2}$ has been reached, the amount of probe available for additional hybridization to the filter is negligible. For probes that do not have the capacity to self-anneal (e.g., RNA probes synthesized in vitro by bacteriophage-encoded DNA-dependent RNA polymerases; see Chapter 10), the hybridization time may be shortened, since the lack of a competing reaction in the solution favors hybridization of the probe to the DNA immobilized on the filter.

5. Several different types of agents can be used to block the nonspecific attachment of the probe to the surface of the filter. These include Denhardt's reagent (Denhardt 1966), heparin (Singh and Jones 1984), and nonfat dried milk (Johnson et al. 1984). Frequently, these agents are used in combination with denatured, fragmented salmon sperm or yeast DNA and detergents such as SDS. In our experience, virtually complete suppression of background hybridization is obtained by pre-hybridizing filters with a blocking agent consisting of $5 \times$ Denhardt's reagent, 0.5% SDS, and 100 μ g/ml denatured, fragmented DNA. We recommend this mixture whenever the signal-to-noise ratio is expected to be low, for example, when carrying out northern analysis of low-abundance mRNAs or Southern hybridizations with single-copy sequences of mammalian DNA. However, in most other circumstances (Grunstein/Hogness hybridization [1975], Benton/Davis hybridization [1977], Southern hybridization [1975] of abundant DNA sequences, etc.), we recommend using 0.25% nonfat dried milk ($0.05 \times$ BLOTTO; Johnson et al. 1984). This is much less expensive, easier to use than Denhardt's reagent, and, for these purposes, gives results that are equally satisfactory. In general, Denhardt's reagent is more effective for nylon membranes. The signal-to-noise ratio obtained with most brands of nylon membranes is higher with Denhardt's reagent than with BLOTTO. Nonfat dried milk is not recommended when using RNA probes or when carrying out northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase activity. For more information about blocking agents, see Table 9.1.
6. Blocking agents are usually included in both the prehybridization and hybridization solutions when nitrocellulose filters are used. However, when the nucleic acid is immobilized on nylon membranes, the blocking agents are often omitted from the hybridization solution, since high

TABLE 9.1 Blocking Agents Used to Suppress Background in Hybridization Experiments

Agent	Recommended uses
Denhardt's reagent	northern hybridizations hybridizations using RNA probes single-copy Southern hybridizations hybridizations involving DNA immobilized on nylon membranes

Denhardt's reagent (Denhardt 1966) is usually made up as a 50× stock solution, which is filtered and stored at -20°C. The stock solution is diluted tenfold into prehybridization buffer (usually 6× SSC or 6× SSPE containing 0.5% SDS and 100 µg/ml denatured, fragmented, salmon sperm DNA). 50× Denhardt's reagent contains 5 g of Ficoll (Type 400, Pharmacia) 5 g of polyvinylpyrrolidone, 5 g of bovine serum albumin (Pentex Fraction V), and H₂O to 500 ml.

BLOTTO	Grunstein/Hogness hybridization Benton/Davis hybridization all Southern hybridizations other than single-copy dot blots
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1× BLOTTO (Bovine Lacto Transfer Technique Optimizer; Johnson et al. 1984) is 5% non-fat dried milk dissolved in water containing 0.02% sodium azide. It should be stored at 4°C and diluted 25-fold into hybridization buffer before use. BLOTTO should not be used in combination with high concentrations of SDS, which will cause the milk proteins to precipitate. If background hybridization is a problem, NP-40 may be added to the hybridization solution to a final concentration of 1%. BLOTTO should not be used as a blocking agent in northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase.

Caution: Sodium azide is poisonous. It should be handled with great care, wearing gloves, and solutions containing it should be clearly marked.

Heparin	Southern hybridization in situ hybridization
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Heparin (Sigma H 7005 porcine grade II or equivalent) is dissolved at a concentration of 50 mg/ml in 4× SSPE or 4× SSC and stored at 4°C. It is used as a blocking agent at a concentration of 500 µg/ml in hybridization solutions containing dextran sulfate; in hybridization solutions that do not contain dextran sulfate, heparin is used at a concentration of 50 µg/ml (Singh and Jones, 1984).

Denatured, fragmented salmon sperm DNA	Southern and northern hybridizations
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Salmon sperm DNA (Sigma type III sodium salt) is dissolved in water at a concentration of 10 mg/ml. If necessary, the solution is stirred on a magnetic stirrer for 2–4 hours at room temperature to help the DNA to dissolve. The solution is adjusted to 0.1 M NaCl and extracted once with phenol and once with phenol:chloroform. The aqueous phase is recovered and the DNA is sheared by passing it 12 times rapidly through a 17-gauge hypodermic needle. The DNA is precipitated by adding 2 volumes of ice-cold ethanol. It is then recovered by centrifugation and redissolved at a concentration of 10 mg/ml in water. The OD₂₆₀ of the solution is determined and the exact concentration of the DNA is calculated. The solution is then boiled for 10 minutes and stored at -20°C in small aliquots. Just before use, the solution is heated for 5 minutes in a boiling-water bath and then chilled quickly in ice water. Denatured, fragmented salmon sperm DNA should be used at a concentration of 100 µg/ml in hybridization solutions.

concentrations of protein are believed to interfere with the annealing of the probe to its target. This quenching of the hybridization signal is particularly noticeable when oligonucleotides or probes less than 100 nucleotides in length are used.

7. In the presence of 10% dextran sulfate or 10% polyethylene glycol, the rate of hybridization is accelerated approximately tenfold (Wahl et al. 1979; Rerz and Kurz 1984; Amasino 1986) because nucleic acids are excluded from the volume of the solution occupied by the polymer and their effective concentration is therefore increased. Although dextran sulfate and polyethylene glycol are useful in circumstances where the rate of hybridization is the limiting factor in detecting rare sequences (e.g., northern or genomic Southern blots), they are of no benefit when screening bacterial colonies or bacterial plaques. In addition, they can sometimes lead to high backgrounds, and hybridization solutions containing them are always difficult to handle because of their viscosity. We therefore recommend that dextran sulfate and polyethylene glycol not be used unless the rate of hybridization is very slow, the filter contains very small amounts of DNA, or the amount of radiolabeled probe is limiting.
8. To maximize the rate of annealing of the probe with its target, hybridizations are usually carried out in solutions of high ionic strength ($6 \times$ SSC or $6 \times$ SSPE) at a temperature that is $20-25^{\circ}\text{C}$ below the melting temperature (T_m). Both solutions work equally well when hybridization is carried out in aqueous solvents. However, when formamide is included in the hybridization buffer, $6 \times$ SSPE is preferred because of its greater buffering power.
9. In general, the washing conditions should be as stringent as possible (i.e., a combination of temperature and salt concentration should be chosen that is approximately $12-20^{\circ}\text{C}$ below the calculated T_m of the hybrid under study). The temperature and salt conditions can often be determined empirically in preliminary experiments in which samples of genomic DNA immobilized on filters are hybridized to the probe of interest and then washed under conditions of different stringencies.
10. To minimize background problems, it is best to hybridize for the shortest possible time using the minimum amount of probe. For Southern hybridization of mammalian genomic DNA where each lane of the gel contains $10 \mu\text{g}$ of DNA, $10-20 \text{ ng/ml}$ radiolabeled probe (sp. act. = 10^9 cpm/ μg or greater) should be used and hybridization should be carried out for 12-16 hours at 68°C in aqueous solution or for 24 hours at 42°C in 50% formamide. For Southern hybridization of fragments of cloned DNA where each band of the restriction digest contains 10 ng of DNA or more, much less probe is required. Typically, hybridization is carried out for 6-8 hours using $1-2 \text{ ng/ml}$ radiolabeled probe (sp. act. = 10^9 cpm/ μg or greater).
11. *Useful facts:*
 - a. The T_m of the hybrid formed between the probe and its target may be estimated from the following equation (Bolton and McCarthy 1962):

9.50 Analysis and Cloning of Eukaryotic Genomic DNA

$$T_m = 81.5^\circ\text{C} - 16.6(\log_{10}[\text{Na}^+]) + 0.41(\% \text{G} + \text{C}) - 0.63(\% \text{formamide}) - (600/l)$$

where l = the length of the hybrid in base pairs.

This equation is valid for:

- Concentrations of Na^+ in the range of 0.01 M to 0.4 M. It predicts T_m less accurately in solutions of higher $[\text{Na}^+]$.
- DNAs whose G + C content is in the range of 30% to 75%. Note that the depression of T_m in solutions containing formamide is greater for poly(dA:dT) ($0.75^\circ\text{C}/1\%$ formamide) and less for DNAs rich in poly(dG:dC) ($0.50^\circ\text{C}/1\%$ formamide) (Casey and Davidson 1977).

The equation applies to the "reversible" T_m that is defined by optical measurement of hyperchromicity at OD_{257} . The "irreversible" T_m , which is more important for autoradiographic detection of DNA hybrids, is usually $7\text{--}10^\circ\text{C}$ higher than that predicted by the equation.

Similar equations have been derived for:

- RNA probes hybridizing to immobilized RNA (Bodkin and Knudson 1985)

$$T_m = 79.8^\circ\text{C} + 18.5(\log_{10}[\text{Na}^+]) + 0.58(\% \text{G} + \text{C}) + 11.8(\% \text{G} + \text{C})^2 - 0.35(\% \text{formamide}) - (820/l)$$

- DNA:RNA hybrids (Casey and Davidson 1977)

$$T_m = 79.8^\circ\text{C} + 18.5(\log_{10}[\text{Na}^+]) + 0.58(\% \text{G} + \text{C}) + 11.8(\% \text{G} + \text{C})^2 - 0.50(\% \text{formamide}) - (820/l)$$

Comparison of these equations shows that the relative stability of nucleic acid hybrids decreases in the following order: RNA:RNA (most stable), RNA:DNA (less stable), and DNA:DNA (least stable). In aqueous solutions, the T_m of a DNA:DNA hybrid is approximately 10°C lower than that of the equivalent RNA:RNA hybrid. In 80% formamide, the T_m of an RNA:DNA hybrid is approximately 10°C higher than that of the equivalent DNA:DNA hybrid.

- The T_m of a double-stranded DNA decreases by $1\text{--}1.5^\circ\text{C}$ with every 1% decrease in homology (Bonner et al. 1973).

The above equations apply only to hybrids greater than 100 nucleotides in length. The behavior of oligonucleotide probes is described in detail in Chapter 11.

For a general discussion of hybridization of nucleic acids bound to solid supports, see Meinkoth and Wahl (1984).

Hybridization of Radiolabeled Probes to Nucleic Acids Immobilized on Nitrocellulose Filters or Nylon Membranes

Although the method given below deals with RNA or DNA immobilized on nitrocellulose filters, only slight modifications are required to adapt the procedure to nylon membranes. These modifications are noted at the appropriate places in the text.

1. Prepare the prehybridization solution appropriate for the task at hand. Approximately 0.2 ml of prehybridization solution will be required for each square centimeter of nitrocellulose filter or nylon membrane.

The prehybridization solution should be filtered through a 0.45-micron disposable cellulose acetate filter (Schleicher and Schuell Uniflow syringe filter No. 57240 or equivalent).

Prehybridization solutions

For detection of low-abundance sequences:

Either

6 × SSC (or 6 × SSPE)

5 × Denhardt's reagent

0.5% SDS

100 µg/ml denatured, fragmented salmon sperm DNA

or

6 × SSC (or 6 × SSPE)

5 × Denhardt's reagent

0.5% SDS

100 µg/ml denatured, fragmented salmon sperm DNA

50% formamide

For preparation of Denhardt's reagent and denatured, fragmented salmon sperm DNA, see Table 9.1.

Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by stirring on a magnetic stirrer with Dowex XG8 mixed-bed resin for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at -70°C.

For detection of moderate- or high-abundance sequences:

Either

6 × SSC (or 6 × SSPE)

0.05 × BLOTTO

or

6 × SSC (or 6 × SSPE)

0.05 × BLOTTO

50% formamide

For preparation of BLOTTO, see Table 9.1.

9.52 Analysis and Cloning of Eukaryotic Genomic DNA

When ^{32}P -labeled cDNA or RNA is used as a probe, poly(A)⁺ RNA at a concentration of 1 $\mu\text{g}/\text{ml}$ may be included in the prehybridization and hybridization solutions to prevent the probe from binding to T-rich sequences that are found fairly commonly in eukaryotic DNA.

2. Float the nitrocellulose filter or nylon membrane containing the target DNA on the surface of a tray of $6 \times \text{SSC}$ (or $6 \times \text{SSPE}$) until it becomes thoroughly wetted from beneath. Submerge the filter for 2 minutes.
3. Slip the wet filter into a heat-sealable bag (e.g., Sears Seal-A-Meal or equivalent). Add 0.2 ml of prehybridization solution for each square centimeter of nitrocellulose filter or nylon membrane. Squeeze as much air as possible from the bag. Seal the open end of the bag with the heat sealer. Incubate the bag for 1–2 hours submerged at the appropriate temperature (68°C for aqueous solvents; 42°C for solvents containing 50% formamide).

Often, small bubbles of air form on the surface of the filter as the temperature of the prehybridization solution increases. It is important that these bubbles be removed by occasionally agitating the fluid in the bag; otherwise, the components of the prehybridization solution will not be able to coat the filter evenly. This problem can be minimized by heating the prehybridization solution to the appropriate temperature before adding it to the bag.

4. If the radiolabeled probe is double-stranded, denature it by heating for 5 minutes at 100°C . Single-stranded probe need not be denatured. Chill the probe rapidly in ice water.

Alternatively, the probe may be denatured by adding 0.1 volume of 3 N NaOH. After 5 minutes at room temperature, transfer the probe to ice water and add 0.05 volume of 1 M Tris · Cl (pH 7.2) and 0.1 volume of 3 N HCl. Store the probe in ice water until it is needed.

For Southern hybridization of mammalian genomic DNA where each lane of the gel contains 10 μg of DNA, 10–20 ng/ml radiolabeled probe (sp. act. = 10^5 cpm/ μg or greater) should be used. For Southern hybridization of fragments of cloned DNA where each band of the restriction digest contains 10 ng of DNA or more, much less probe is required. Typically, hybridization is carried out for 6–8 hours using 1–2 ng/ml radiolabeled probe (sp. act. = 10^5 cpm/ μg or greater).

5. Working quickly, remove the bag containing the filter from the water bath. Open the bag by cutting off one corner with scissors. Add the denatured probe to the prehybridization solution, and then squeeze as much air as possible from the bag. Reseal the bag with the heat sealer so that as few bubbles as possible are trapped in the bag. To avoid radioactive contamination of the water bath, the resealed bag should be sealed inside a second, noncontaminated bag.

When using nylon membranes, the prehybridization solution should be *completely* removed from the bag and immediately replaced with hybridization solution. The probe is then added and the bag is resealed.

Hybridization solution for nylon membranes

6 × SSC (or 6 × SSPE)

0.5% SDS

100 µg/ml denatured, fragmented salmon sperm DNA

50% formamide (if hybridization is to be carried out at 42°C)

6. Incubate the bag submerged in a water bath set at the appropriate temperature for the required period of hybridization.

7. Wearing gloves, remove the bag from the water bath and immediately cut off one corner. Pour out the hybridization solution into a container suitable for disposal, and then cut the bag along the length of three sides. Remove the filter and immediately submerge it in a tray containing several hundred milliliters of 2 × SSC and 0.5% SDS at room temperature.

Important: Do not allow the filter to dry out at any stage during the washing procedure.

8. After 5 minutes, transfer the filter to a fresh tray containing several hundred milliliters of 2 × SSC and 0.1% SDS and incubate for 15 minutes at room temperature with occasional gentle agitation.

If short oligonucleotides are used as probes, washing should be carried out only for brief periods (1–2 minutes) at the appropriate temperature. For a discussion of the stability of hybrids involving oligonucleotides, see Chapter 11.

9. Transfer the filter to a flat-bottom plastic box containing several hundred milliliters of fresh 0.1 × SSC and 0.5% SDS. Incubate the filter for 30 minutes to 1 hour at 37°C with gentle agitation.

10. Replace the solution with fresh 0.1 × SSC and 0.5% SDS, and transfer the box to a water bath set at 68°C for an equal period of time. Monitor the amount of radioactivity on the filter using a hand-held minimonitor. The parts of the filter that do not contain DNA should not emit a detectable signal. You should not expect to pick up a signal on the minimonitor from filters containing mammalian DNA that has been hybridized to single-copy probes.

11. Briefly wash the filter with 0.1 × SSC at room temperature. Remove most of the liquid from the filter by placing it on a pad of paper towels.

12. Place the damp filter on a sheet of Saran Wrap. Apply adhesive dot labels marked with radioactive ink to several asymmetric locations on the Saran Wrap. These markers serve to align the autoradiograph with the filter. Cover the labels with Scotch Tape. This prevents contamination of the film holder or intensifying screen with the radioactive ink.

Radioactive ink is made by mixing a small amount of ³²P with waterproof black drawing ink. We find it convenient to make the ink in three grades: very hot

(>2000 cps on a hand-held minimonitor), hot (>500 cps on a hand-held minimonitor), and cool (>50 cps on a hand-held minimonitor). Use a fiber-tip pen to apply ink of the desired hotness to the adhesive labels. Attach radioactive-warning tape to the pen, and store it in an appropriate place.

13. Cover the filter with a second sheet of Saran Wrap, and expose the filter to X-ray film (Kodak XAR-2 or equivalent) to obtain an autoradiographic image (see Appendix E). The exposure time should be determined empirically. However, single-copy sequences in mammalian genomic DNA can usually be detected after 16-24 hours of exposure at -70°C with an intensifying screen.

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